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[26] are extremely powerful methods
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'v into a genetic fusion with other
·binding domains, modified toxin
:Fvs, has the potential to generate
ns.[27-29]

9, 293 (1991).
. Moller, *Proc. Natl. Acad. Sci. U.S.A.* **85**,

Wallace, M. Abrhamson, K. Fry, and C.

D. J. Chiswell, *Nature (London)* **348**, 552

Benkovic, R. A. Lerner, E. D. Getzoff, and
654 (1990).

[5] Humanization of Monoclonal Antibodies

By DETLEF GÜSSOW and GERHARD SEEMANN

Since Köhler and Milstein's breakthrough in hybridoma technology,[1] monoclonal antibodies (MAbs) have become ever more important tools in all fields of biology and medicine. A large panel of diagnostic as well as therapeutic MAbs have been developed that are of potential value for *in vivo* diagnosis and therapy in humans.[2,3] Virtually all MAbs are of rodent origin and exposure of humans to such heterologous MAbs can severely limit their use because of allergic reactions. Immunocompetent individuals may develop human anti-mouse immunoglobulin (Ig) antibodies (HAMA).[4,5] Human MAbs would obviously eliminate such difficulties but the technology to produce them will require considerable refinement before it becomes generally applicable.[6] However, standard molecular biology techniques can offer a way out of the dilemma. Working on the level of the immunoglobulin genes, we are now able to recombine variable-region genes of the heavy or light chains (V_H or V_L) with constant-region genes of any desired isotype, and, more importantly, we also have the choice between constant-region genes of different species.[7,8] Shuffling the variable domains between different antibody genes is now an established technique for creating so-called chimeric antibodies where the variable domain is of murine origin and the constant region is human. These chimeric MAbs usually resemble their parental mouse MAb in specificity as well as affinity for the antigen.[9,10] Chimeric MAbs are already more suitable for therapeutic use in humans. However, the mouse variable region still carries a host of immunogenic epitopes and it is likely that chimeric MAbs are still immu-

[1] G. Köhler and C. Milstein, *Nature (London)* **256**, 495 (1975).
[2] J. L. Murray and M. W. Unger, *Crit. Rev. Oncol./Hematol.* **8**, 227 (1988).
[3] H.-H. Sedlacek, G. Schulz, A. Steinsträsser, L. Kuhlmann, A. Schwarz, L. Seidel, G. Seemann, H.-P. Kraemer, and K. Bosslet, *in* "Contributions to Oncology" (S. Eckhardt, J. H. Holzner and G. A. Nagel, eds.), Vol. 32. Karger, Basel, 1988.
[4] R. A. Miller, A. R. Oseroff, P. T. Stratte, and R. Levy, *Blood* **62**(5), 988 (1983).
[5] M. J. P. G. Van Kroonenburgh and E. K. J. Pauwels, *Nucl. Med. Commun.* **9**, 919 (1988).
[6] J. E. Boyd, K. James, and D. B. L. McClelland, *Trends Biotechnol.* **2**(3), 70 (1984).
[7] V. T. Oi, S. L. Morrison, L. A. Herzenberg, and P. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 825 (1983).
[8] S. L. Morrison and V. T. Oi, *Adv. Immunol.* **44**, 65 (1989).
[9] G. L. Boulianne, N. Hozumi, and M. J. Shulman, *Nature (London)* **312**, 643 (1984).
[10] S. L. Morrison, M. J. Johnson, L. A. Herzenberg, and V. T. Oi, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6851 (1984).

nogenic as it has been shown in a mouse model system with chimeric MAbs containing murine V and human constant domains.[11]

Jones *et al.*[12] and Riechmann *et al.*[13] have taken another more subtle approach, in order to make a more human form of a rodent MAb. Antigen-binding sites, composed of the three CDRs (complementarity determining regions) of the heavy chain and the three CDRs of the light chain, can be taken from a rodent MAb and inserted directly into the framework of a human antibody, thus transplanting only the CDRs rather than the entire variable domain of a rodent antibody. This is achieved by the use of oligonucleotides to replace the CDRs of an appropriate human immunoglobulin gene with the CDRs from a rodent MAb with a desired specificity. The CDRs or hypervariable regions are unlikely to carry any species-specific characteristics, and therefore such a "reshaped" human antibody should be indistinguishable from genuine human immunoglobulins. Although anti-idiotypic antibodies against a reshaped or "humanized" MAb can be generated *in vivo,* the occurrence of such antibodies does not seem to be a problem in therapy (H. Waldmann, personal communication, 1989).

The general applicability of the antibody-reshaping technique relies on two assumptions: (1) the antigen-binding site is fashioned by the CDRs as defined by Kabat *et al.*,[14] no other parts of the variable region taking part in binding the antigen; (2) the frameworks of the variable domains serve as a scaffold to support the CDRs in a specific way that facilitates antigen binding. Subsequently it is of great importance to retain the interactions between the donor (rodent) CDRs and the acceptor (human) framework as closely as possible to the CDR–framework interactions of the original MAb.

However, the affinity of the first fully reshaped antibody, CAMPATH1, was nearly 40-fold lower compared to the original rat MAb.[13] Close inspection of the sequences of the hypervariable loops in the human and rat antibodies, in particular at their junctions with the framework regions of the V_H domain, showed the possible origin of the flaw. A comparison of the amino acid sequence in the flanking region of CDR1 (residues 31–35, Kabat *et al.*[14]) showed that the original rat antibody has a phenylalanine at

[11] M. Brüggemann, G. Winter, H. Waldmann, and M. S. Neuberger, *J. Exp. Med.* **170,** 2153 (1989).

[12] P. T. Jones, P. H. Dear, J. Foote, M. S. Neuberger, and G. Winter, *Nature (London)* **321,** 522 (1986).

[13] L. Riechmann, M. Clark, H. Waldmann, and G. Winter, *Nature (London)* **332,** 323 (1988).

[14] E. A. Kabat, T. T. Wu, M. Reid-Miller, H. M. Perry, and K. S. Gottesman, "Sequences of Proteins of Immunological Interest," 4th Ed., U.S. Department of Health and Human Services, US Washington, D.C., 1987.

se model system with chimeric
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have taken another more subtle
in form of a rodent MAb. Anti-
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rted directly into the framework
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ly. This is achieved by the use of
in appropriate human immuno-
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a "reshaped" human antibody
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reshaped or "humanized" MAb
of such antibodies does not seem
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dy-reshaping technique relies on
site is fashioned by the CDRs as
the variable region taking part in
f the variable domains serve as a
ific way that facilitates antigen
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t antibody has a phenylalanine at

M. S. Neuberger, *J. Exp. Med.* **170,** 2153

er, and G. Winter, *Nature (London)* **321,**

Winter, *Nature (London)* **332,** 323 (1988).
rry, and K. S. Gottesman, "Sequences of
J.S. Department of Health and Human

position 27 (phenylalanine and tyrosine are the most common amino acids at this position). Existing structural data of the human myeloma protein KOL show that Phe-27 packs against residues 32 and 34 to support CDR1.[15] The NEW framework (the human acceptor for the CDR transplants of the heavy chain)[13] has a serine at position 27 and thus fails to support the CDR in the same way as in the original rat antibody. A subsequent engineered mutation, serine to phenylalanine at position 27, restored the binding affinity of the humanized CAMPATH1 antibody close to the original affinity. This example demonstrates that amino acids in the framework, in particular those that are located close to the CDRs, must be considered. We now routinely compare the sequences of the mouse hybridoma and the human target variable sequences with each other and their respective family consensus sequences.

Here we describe the humanization of the murine monoclonal antibody BW431/26,[16] which has binding specificity for carcinoembryonic antigen (CEA) and is presently used as a murine MAb for immunoscintigraphy of CEA-producing tumors such as colorectal, breast, and lung carcinomas.[17]

Cloning of Immunoglobulin V-Region Genes

A prerequisite for the humanization of MAb is knowledge of the nucleotide sequences of the V-region genes. To date the most elegant way to clone and sequence Ig V-region genes has been described by Orlandi and colleagues.[18] They used the polymerase chain reaction (PCR)[19] to amplify specifically Ig V-region genes and cloned the amplified DNA fragments into vectors that allow easy sequencing and expression. To do this they identified conserved regions at each end of the nucleotide sequences encoding V domains of mouse Ig heavy (V_H) and light (V_L) chains by comparing the frequencies of the most common nucleotides in V_H and V_L gene sequences.[14] Since oligonucleotide primers used in PCR do not need to match their target sequence exactly[20] they were able to design oligonu-

[15] M. Marquart, J. Deisenhofer, and R. Huber, *J. Mol. Biol.* **141,** 369 (1980).
[16] K. Bosslet, A. Steinsträsser, A. Schwarz, H. P. Harthus, G. Lüben, L. Kuhlmann, and H. H. Sedlacek, *Eur. J. Nucl. Med.* **14,** 523 (1988).
[17] R. P. Baum, A. Hertel, M. Lorenz, A. Schwarz, A. Encke, and G. Hör, *Nucl. Med. Commun.* **10,** 345 (1989).
[18] R. Orlandi, D. H. Güssow, P. T. Jones, and G. Winter, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 3833 (1989).
[19] R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim, *Science* **230,** 1350 (1985).
[20] C. C. Lee, X. Wu, R. A. Gibbs, R. G. Cook, D. M. Muzny, and C. T. Caskey, *Science* **239,** 1288 (1988).

cleotides that map to the 5′ and 3′ regions of V_H and V_L genes and contain restriction sites for subsequent forced cloning into suitable vectors (Fig. 1 and Ref. 19).

The amplified V_H and V_L genes cover the V gene exons only. For expression in a mammalian system it is necessary to bring them into genomic configuration. The V_H and V_L genes are force cloned into KS^+ vectors (pBluescript II KS^+; Stratagene, La Jolla, CA) containing the 5′ region of immunoglobulin heavy and light chain genes, respectively (including promoter, signal exon, and irrelevant V gene exon) as a unique *Hin*dIII/*Bam*HI restriction fragment (Fig. 2). The endogenous V gene exons contain the same or compatible restriction sites as the amplification primers in the appropriate positions and are exchanged for the amplified V genes of the hybridoma by forced cloning (see the section, Cloning of V Genes). These vectors can be used for nucleotide sequence determination of the specific V genes and for forced cloning of the amplified V_H and V_L cassettes into expression vectors that contain Ig constant-region genes, using the *Hin*dIII and *Bam*HI restriction sites (Fig. 3).

Mouse V_H Forward:

5′ TGAGGAGACGGTGACCGTGGTCCCTTGGCC 3′
Bst E II

Mouse V_H Backward:

5′ AGGT(C/G)(C/A)A(G/A)(G/A)CTGCAG(G/C)AGTC(T/A)GG 3′
Pst I

Mouse V_K Forward:

5′ GTTAGATCTCCAGCTTGGTCCC 3′
Bgl II

Mouse V_K Backward:

5′ GACATTCAGCTGACCCAGTCTCCA 3′
Pvu II

FIG. 1. Oligonucleotide primer for the amplification of V_H and V_K genes. The mouse V_H backward primer is a mixture of 32 primers. The locations of the restriction sites are indicated.

V_H and V_L genes and contain
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(see the section, Cloning of V
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iTCCCTTGGCC 3′

CAG(G/C)AGTC(T/A)GG 3′
:1

CCC 3′

TCTCCA 3′

ɔn of V_H and V_K genes. The mouse V_H
: locations of the restriction sites are

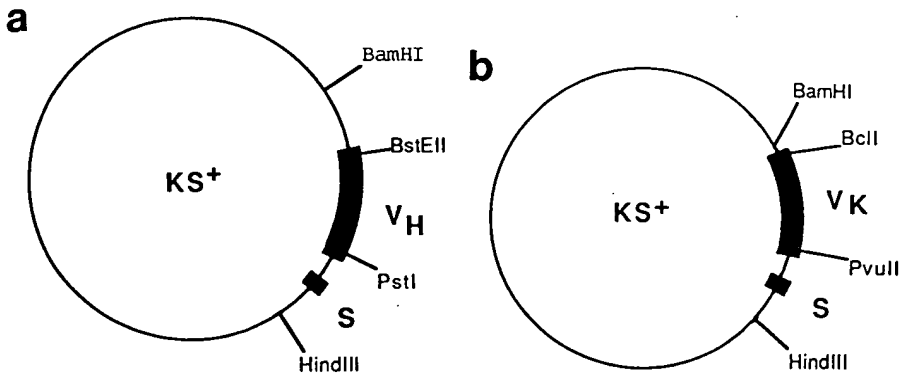


FIG. 2. Restriction maps and intron/exon organization of the V gene inserts of the KS+ plasmids containing a V_H (a) or a V_L (b) gene. The inserts are flanked by *Bam*HI and *Hin*dIII restriction sites. Exons are represented by black bars. S, Signal exon; V_H/V_K, V gene exons. The restriction sites for cloning of the amplified V genes are indicated. The *Bcl*I restriction site in the V_K exon is compatible with *Bgl*II.

Preparation of mRNA

Poly(A)+ mRNA is prepared following one of the standard procedures. An example is as follows:

Centrifuge hybridoma cells (825 *g*; room temperature; 15 min)
Discard supernatant
Resuspend in 100 ml cold phosphate-buffered saline (PBS) (0°)
Centrifuge (275 *g*; room temperature; 10 min)
Discard supernatant
Resuspend in 100 ml cold PBS
Centrifuge as above
Discard supernatant
Add 22 ml lysis buffer to the pellet, vortex for 5 sec, incubate on ice (5 min)
Centrifuge nuclei (1630 *g*; 4°; 15 min)
Transfer supernatant to new tube and add 25 ml 2× proteinase K, buffer
Incubate at 50° for 60 min
Add 0.1 vol 5 *M* NaCl and let cool down to room temperature
Apply the solution to an oligo(dT)-Sepharose column (1-ml) column volume equilibrated with binding buffer)
Wash with 10 ml binding buffer and 15 ml washing buffer
Elute poly(A)+ mRNA with 7.5 ml elution buffer and collect three fractions of 2.5 ml
Add 0.1 vol of 3 *M* sodium acetate and 2.5 vol ethanol to each fraction
Store at −20°

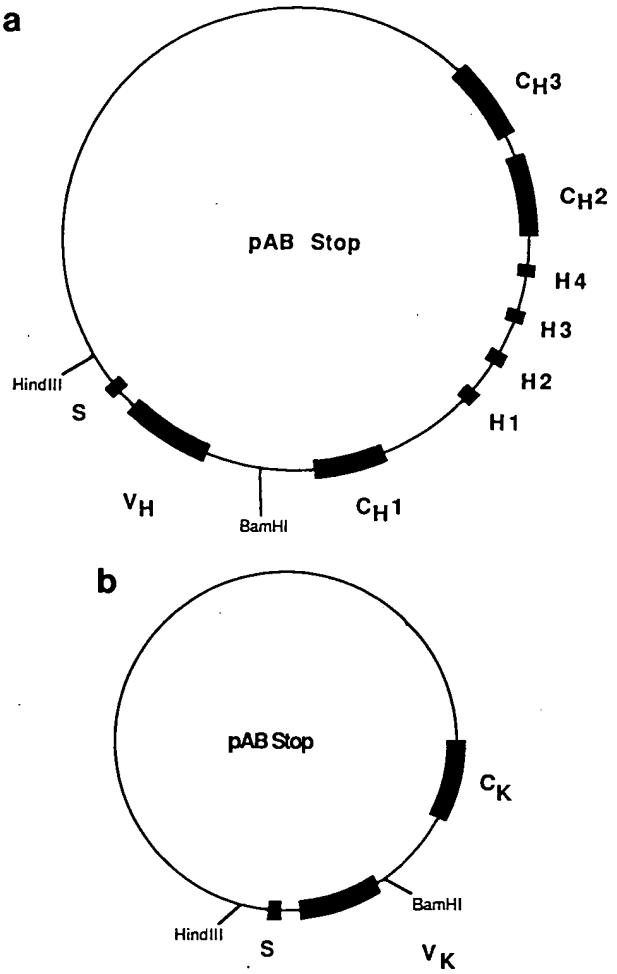
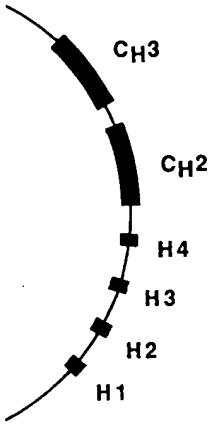


FIG. 3. Restriction maps and intron/exon organization of the expression vectors containing the human (a) heavy (IgG_3) and (b) light chain (κ) constant-region genes. The intrinsic V genes are exchanged for the hybridoma V genes using the *Hin*dIII and *Bam*HI restriction sites. Exons are represented by black bars. S, Signal exon; V_H/V_K, V gene exons; C_H1, C_H2, C_H3, and C_K, constant-domain exons; H1, H2, H3, and H4, hinge region exons.

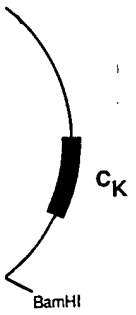
The average yield is approximately 40 μg of poly(A)$^+$ mRNA per 1×10^8 hybridoma cells.

Lysis buffer: 0.14 *M* NaCl, 2.0 m*M* $MgCl_2$, 10.0 m*M* Tris, pH 8.6, 0.5% Nonidet P-40 (NP-40)

Proteinase K buffer (2×): 200 m*M* Tris, pH 7.4, 300 m*M* NaCl, 25 m*M*



1



V_K

ion of the expression vectors contain-
constant-region genes. The intrinsic V
g the *Hin*dIII and *Bam*HI restriction
on; V_H/V_K, V gene exons; C_H1, C_H2,
d H4, hinge region exons.

f poly(A)$^+$ mRNA per 1×10^8

, 10.0 m*M* Tris, pH 8.6, 0.5%

H 7.4, 300 m*M* NaCl, 25 m*M*

ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), 2 mg/ml proteinase K

Binding buffer: 0.5 *M* NaCl, 10.0 m*M* Tris, pH 7.8, 5.0 m*M* EDTA, 0.5% SDS

Washing buffer: 0.15 *M* NaCl, 10.0 m*M* Tris, pH 7.8, 5.0 m*M* EDTA, 0.5% SDS

Elution buffer: No NaCl, 10.0 m*M* Tris, pH 7.8, 5.0 m*M* EDTA, 0.5% SDS

Amplification and Cloning of V Genes

Amplification of the V genes can be performed either by synthesis of first-strand cDNA followed by polymerase chain reaction (PCR) or by PCR directly from the poly(A)$^+$ mRNA.[21]

First-Strand cDNA Synthesis. Mix in the following order:

Reverse transcriptase buffer (5×)	10.0 μl
Dithiothreitol (DTT) (0.1 *M*)	2.5 μl
Human placental ribonuclease inhibitor (50 U/ul)	3.0 μl
dNTPs (10 m*M*)	5.0 μl
Primer (10 p*M*/μl)	5.0 μl
Poly(A)$^+$ mRNA (5–10 μg)	
ddH_2O	to 47.0 μl

Mix gently and centrifuge for a few seconds, then add

Reverse transcriptase (25 U/μl)	3.0 μl

Incubate at 42° for 1 hr.

PCR Reaction from cDNA

H_2O:	29.5 μl
dNTPs (2.5 m*M*):	5.0 μl
PCR buffer (10×):	5.0 μl
Oligonucleotide primers (5 p*M*/μl):	5.0 μl
Taq polymerase (The Perkin-Elmer Corp., Emmeryville, CA) (2.5 U/μl)[21a]	0.5 μl
	0.5 μl

At this point the reaction mix can be treated for 5 min with UV light (254 or 300 nm) to avoid contamination (for example, with previously amplified DNA).

[21] W. T. Tse and B. G. Forget, *Gene* **88**, 293 (1990).

[21a] In case of the V_H primers the Taq polymerase was added to the reaction mixture after the first denaturation and annealing steps to avoid primer dimer formation.

Add 5.0 μl of first-strand cDNA synthesis reaction and seal the surface of the reaction mixture with a drop of paraffin oil. Amplify for 30 to 50 cycles. The standard programs we use are the following: 94°, 1 min; 52° (for V_K, V_λ) or 57° (for V_H), 1 min; 72° for 2 min.

Analyze 5 μl of the reaction mixture on a 2% agarose gel.

PCR Reaction from mRNA. For PCR for mRNA resuspend 1–5 μg of poly(A)$^+$ mRNA in 50 μl PCR reaction mix and proceed as described above for the PCR from cDNA.

If the amplification reactions are unsuccessful under these conditions, the addition of 5.0 μl Perfect Match (Stratagene) and a modification of the annealing and extention conditions as well as $MgCl_2$ concentrations may improve the results.

Cloning of V Genes

If the amplification is successful, the remaining 45 μl of the reaction mixture is purified with Geneclean (BIO 101, Inc., La Jolla, CA). The purified amplification products (in 20 μl doubly distilled H_2O) are treated as follows: 10 μl is double digested with *Pst*I/*Bst*EII for the V_H gene or *Pvu*II/*Bgl*II for the V_L gene. The digested samples are analysed on a 2% TAE agarose gel with 3 μl of undigested amplification product as control. The residual amplification product is stored at −20° as the template for the synthesis of additional material, if required.

The vectors for cloning the amplified V genes have been constructed by isolation of the *Hin*dIII/*Bam*HI inserts of the M13 VHPCR and M13 VKPCR vectors described in Orlandi *et al.*[18] into KS$^+$ plasmids. Two internal *Pvu*II sites were removed from the KS$^+$ plasmid backbone prior to the construction of the KS$^+$ VKPCR vector (C. Weber, unpublished data, 1990).

These KS$^+$ vectors (KS$^+$ VHPCR and KS$^+$ VKPCR) are digested with *Pst*I and *Bst*EII (V_H) or *Pvu*II and *Bcl*I (V_L), respectively, and the vector fragment is purified on a 0.8% agarose gel.

Note: The KS$^+$ VKPCR vector was prepared in *Escherichia coli* JM110 (ATCC No. 47013) to avoid dam methylation of the *Bcl*I site.

From the resulting colonies DNA minipreparations are performed and the size of the inserts is analyzed by a *Hin*dIII/*Bam*HI digestion. Clones containing inserts of the correct size (approximately 800 bp for V_H and 650 bp for V_L) are sequenced[22] using primers based 3′ of the CDR3 regions of the V genes (Fig. 4).

Since hybridoma cells may produce more than one heavy or light chain and can contain additional nonfunctional mRNAs it is advisable to clone the isolated V genes into appropriate expression vectors containing Ig C_H

[22] F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).

sis reaction and seal the surface
raffin oil. Amplify for 30 to 50
the following: 94°, 1 min; 52°
ır 2 min.
a 2% agarose gel.
or mRNA resuspend 1–5 μg of
mix and proceed as described

ccessful under these conditions,
igene) and a modification of the
l as $MgCl_2$ concentrations may

remaining 45 μl of the reaction
101, Inc., La Jolla, CA). The
loubly distilled H_2O) are treated
*Pst*I/*Bst*EII for the V_H gene or
l samples are analysed on a 2%
mplification product as control.
red at −20° as the template for
uired.
genes have been constructed by
of the M13 VHPCR and M13
al.[18] into KS^+ plasmids. Two
KS^+ plasmid backbone prior to
or (C. Weber, unpublished data,

KS^+ VKPCR) are digested with
V_L), respectively, and the vector

pared in *Escherichia coli* JM110
ition of the *Bcl*I site.
ipreparations are performed and
*ind*III/*Bam*HI digestion. Clones
oximately 800 bp for V_H and 650
based 3′ of the CDR3 regions of

ore than one heavy or light chain
l mRNAs it is advisable to clone
ression vectors containing Ig C_H

latl. Acad. Sci. U.S.A. **74**, 5463 (1977).

VK/VL: 5′ GGA TCC AAC TGA GGA AGC 3′

VH: 5′ TGT CCC TAG TCC TTC ATG ACC T 3′

FIG. 4. Oligonucleotides for sequencing of the cloned V genes.

and C_L genes and express them in eukaryotic cells (see section entitled "Expression of Humanized MAb"). The resulting chimeric Ig molecules contain the complete mouse V domains and should have the identical antigen-binding properties as the original mouse MAb, if the right pair of V_H and V_L genes has been amplified and cloned.

Reverse transcriptase buffer (5×): 250 m*M* Tris, pH 8.3, 30 m*M* $MgCl_2$, 500 m*M* NaCl
PCR buffer (10×): 100 m*M* Tris, pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.1% (w/v) gelatin
TAE buffer (50×): 2 *M* Tris base, 1 *M* acetic acid, 50 m*M* EDTA
The solutions are autoclaved before use, distributed in 0.5-ml aliquots, and stored at −20°.

Design of Oligonucleotides for Mutagenesis

After determination of the nucleotide sequences of the hybridoma V genes (Fig. 5) they are translated into the corresponding amino acid sequences and compared to the consensus sequences of the murine Ig heavy and light chain V gene families to identify their families of origin (Fig. 6).

Comparison of the translated BW431/26 V_H sequence with the mouse VH consensus sequences marks it as a member of family IA. Likewise BW431/26 V_L is identified as a member of family VI of the κ light chains.

For designing the reshaped V regions, it is necessary to identify amino acid residues that are not conserved or only semiconserved in the framework regions of the human and mouse antibodies in question. Framework regions that are not conserved between human and mouse antibodies, but which might help to shape the CDRs or which might interact directly with the antigen, have to be identified. Unusual amino acid replacements are changes from hydrophilic to hydrophobic, large to small, or change in electrostatic charge. The following amino acid exchanges are considered as conserved replacements and are excluded from the computer inspection: F - Y, L - M, V - I, Q - H, D - E, R - K, W - R, N - D, G - A, S - T, Q - D/E, H - R/N, I - L/M, L - F/V, and V - L/M. The framework and CDR regions are defined by Kabat *et al.* as shown in Fig. 7.

The decision whether framework exchanges must be introduced in addition to the CDR grafting is then made by inspection of the location of the identified amino acid residues in the acceptor V_H or V_K three-dimensional structure.

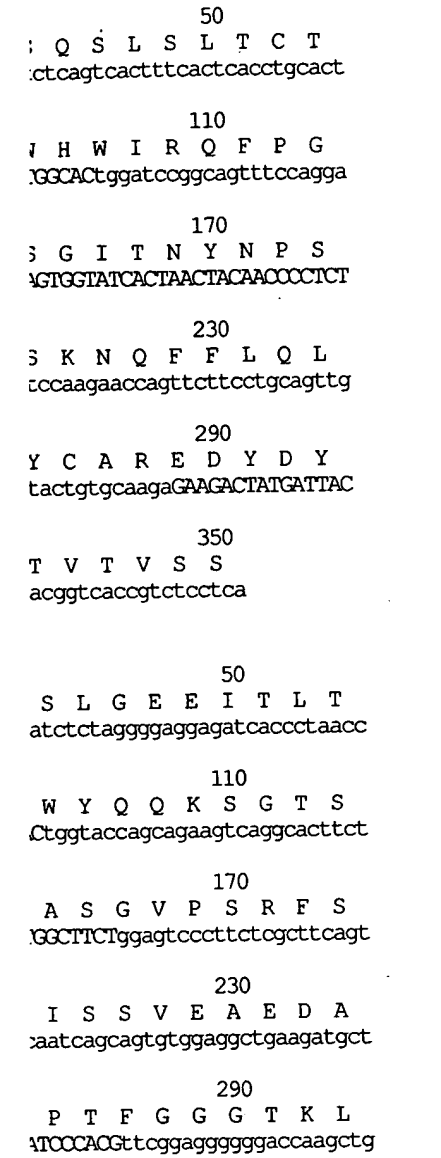
a

```
        10                  30                  50
L  Q  E  S  G  P  D  L  V  K  P  S  Q  S  L  S  L  T  C  T
ctgcaggagtcaggacctgacctggtgaaaccttctcagtcactttcactcacctgcact

        70                  90                  110
V  T  G  Y  S  I  T  S  G  Y  S  W  H  W  I  R  Q  F  P  G
gtcactggctactccatcaccAGTGGTTATAGCTGGCACtggatccggcagtttccagga

        130                 150                 170
N  K  L  E  W  M  G  Y  I  Q  Y  S  G  I  T  N  Y  N  P  S
aacaaactggaatggatgggcTACATACAGTACAGTGGTATCACTAACTACAACCCCTCT

        190                 210                 230
L  K  S  R  I  S  I  T  R  D  T  S  K  N  Q  F  F  L  Q  L
CTCAAAAGTcgaatctctatcactcgagacacatccaagaaccagttcttcctgcagttg

        250                 270                 290
N  S  V  T  T  E  D  T  A  T  Y  Y  C  A  R  E  D  Y  D  Y
aattcagtgactactgaggacacagccacatattactgtgcaagaGAAGACTATGATTAC

        310                 330                 350
H  W  Y  F  D  V  W  G  A  G  T  T  V  T  V  S  S
CACTGGTACTTCGATGTCtggggcgcagggaccacggtcaccgtctcctca
```

b

```
        10                  30                  50
Q  L  T  Q  S  P  A  I  M  S  A  S  L  G  E  E  I  T  L  T
cagctgacccagtctccagcaatcatgtctgcatctctaggggaggagatcaccctaacc

        70                  90                  110
C  S  T  S  S  S  V  S  Y  M  H  W  Y  Q  Q  K  S  G  T  S
tgcAGTACCAGCTCGAGTGTAAGTTACATGCACtggtaccagcagaagtcaggcacttct

        130                 150                 170
P  K  L  L  I  Y  S  T  S  N  L  A  S  G  V  P  S  R  F  S
cccaaactcttgatttatAGCACATCCAACCTGGCTTCTggagtcccttctcgcttcagt

        190                 210                 230
G  S  G  S  G  T  F  Y  S  L  T  I  S  S  V  E  A  E  D  A
ggcagtgggtctgggacctttattctctcacaatcagcagtgtggaggctgaagatgct

        250                 270                 290
A  D  Y  Y  C  H  Q  W  S  S  Y  P  T  F  G  G  G  T  K  L
gccgattattactgcCATCAGTGGAGTAGTTATCCCACGttcggaggggggaccaagctg


E  I
gagatca
```

FIG. 5. Nucleotide sequences of the murine BW431/26 (a) V_H and (b) V_K genes. The sequences start with the *PstI*/*PvuII* restriction sites and continue to the end of the V exon. CDR sequences are typed in capital letters. The amino acid sequences are given in the single-letter code on top of the first base of the triplets.



; BW431/26 (a) V_H and (b) V_K genes. The ites and continue to the end of the V exon. he amino acid sequences are given in the iplets.

A three-step comparison of the framework amino acid residues consists of (1) the donor (mouse) antibody V region with its own V region family consensus sequence, (2) the acceptor (human) antibody V region with its own V region family, and (3) the mouse and human families, which should allow the differences to be dissected in a systematic way, identifying those differences peculiar to the individual antibodies or their entire families.

Heavy Chain V_H

Step 1. The sequence of the (mouse) donor is compared with the consensus sequence for its own family of V_H genes (Fig. 8).

Step 2. The sequence of the (human) acceptor is compared with the consensus sequence for its own family of V_H genes (human V_H II; HuVH.CON2 in Fig. 9).

Step 3. The consensus sequences of the donor (mouse) and acceptor (human) V_H families are compared (Fig. 10).

Light Chain V_K

Step 1. The sequence of the (mouse) donor is compared with the consensus sequence of its own V_K family (MOVK VI) (Fig. 11).

Step 2. The sequence of the (human) acceptor is compared with the consensus of its own V_K family. The human REI V_K sequence is based on Ref. 13. REI is a member of the human V_K family I (HuVKCON SUB1 in Fig. 12).

Step 3. The consensus sequences of the acceptor (human) and donor (mouse) V_K families are compared (Fig. 13).

All unusual amino acid replacements that occur outside the CDRs, as defined by Kabat *et al.*,[14] are marked and the positions at which replacements occur are inspected by computer graphics (Evans & Sutherland Corp., Salt Lake City, UT; Frodo[23]) using as a basis the X-ray structures of NEW[24] and REI.[25] All positions in question are highlighted with a 200% van der Waals radius, to see whether the amino acid residue at a certain position is close to, or actually makes contact with, any of the amino acids composing the CDRs. In such a case an amino acid replacement in the framework of the human V_H or V_K region must be considered.

[23] T. A. Jones, *in* "Computational Crystallography" (D. Sayre, ed.), p. 303. Oxford Univ. Press (Clarendon), London and New York, 1982.

[24] R. J. Poljak, L. M. Amzel, H. P. Avey, B. L. Chen, R. P. Phizackerley, and F. Saul, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3305 (1973).

[25] O. Epp, P. Colman, H. Fehlhammer, W. Bode, M. Schiffer and R. Huber, and W. Palm, *Eur. J. Biochem.* **45**, 513 (1974).

a

```
                          10        20        30        40        50
 1 MOVH IA.CON    EVQLQESGPSLVKPSQTLSLTCSVTGDSITSDYWNXXWIRQFPGNKLEWM
 2 MOVH IB.CON    QVQLKESGPGLVAPSQSLSITCTVSGFSLTSYGVHXSWVRQPPGKGLGTL
 3 MOVH IIA.CON   EVQLQQSGPELVKPGASVKMSCKASGYTFTDYYMKXXWVKQSHGKSLEWI
 4 MOVH IIB.CON   QVQLQQPGAELVKPGASVKLSCKASGYTFTSYWMHXXWVKQRPGQGLEWI
 5 MOVH IIC.CON   EVQLQQSGAELVKPGASVKLSCTASGFNIKDTYMHXXWVKQRPEQGLEWI
 6 MOVH IIIA.CON  EVKLVESGGGLVQPGGSLRLSCATSGFTFSDFYMEXXWVRQPPGKRLEWI
 7 MOVH IIIB.CON  EVKLLESGGGLVQPGGSLKLSCAASGFDFSRYWMSXXWVRQAPGKGLEWI
 8 MOVH IIIC.CON  EVYLEESGGGLVQPGGSMYLSCVASGFTFSNYWMNXXWVRQSPEYGLEWV
10 MOVH VA.CON    EVQLQQSGAELVRAGSSVKMSCKASGYTFTSYGINXXWVKQRPGQGLEWI
11 MOVH VB.CON    EVQLQQSGAELVKAGSSVKMSCSATGYTFSSYGLYXXWVRQAPGQGLEXX
    CONSENSUS    -V-L---G--L----------C---G----------W--------L---

                          60        70        80        90       100
 1 MOVH IA.CON    GYISNXXYSGSTYYNPSLYSRISITRDTSKNQFFLQLNSVTTEDTATYYC
 2 MOVH IB.CON    GVIWXXXAGGSTNYNSALMSRLSISKDNSKSQVFLKMNSLQTDDTAMYYC
 3 MOVH IIA.CON   GDINPXXNNGGTSYNQKFKGKATLTVDKSSSTAYMQLNSLTSEDSAVYYC
 4 MOVH IIB.CON   GRIDPXXNSGGTKYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYC
 5 MOVH IIC.CON   GRIDPXXANGNTKYDPKFQGKATITADTSSNTAYLQLSSLTSEDTAVYYC
 6 MOVH IIIA.CON  AASRNKANDYTTEYSASVKGRFIVSRDTSQSILYLQMNALRAEDTAIYYC
 7 MOVH IIIB.CON  GEINPKADSSTINYTPSLYDYFIISRDNAKNTLYLQMSYVRSEDTALYYC
 8 MOVH IIIC.CON  AEIRLYSHNYATHYAESVYGRFTISRDDSYSSVYLQMNNLRAEDTGIYYC
10 MOVH VA.CON    GYINPXXGNGYTKYNEKFKGKTTLTVDKSSSTAYMQLRSLTSEDSAVYFC
11 MOVH VB.CON    GYISSXXSSAYPNYAQKFQGRVTITADESTNTAYMELSSLRSEDTAVYFC
    CONSENSUS    ------------Y------------D----------------D---Y-C

                         110       120       130       140       150
 1 MOVH IA.CON    ARLYGYYRGDEEDYYAMAFDYWGQGTTXTVSS
 2 MOVH IB.CON    ARDRGVXRYDPDKYFTLWFDYWGQGTLVTVSS
 3 MOVH IIA.CON   ARDYYWYFXXXXXXXYYWFDYWGQGTTVTVSS
 4 MOVH IIB.CON   ARYXYYGSSSXGYXXYXYFDYWGQGTTLTVSS
 5 MOVH IIC.CON   ARGYXXYDXXXXXXXYYAMDYWGQGTSVTVSS
 6 MOVH IIIA.CON  ARDYYGSSXTFGXXXYWYFDVWGAGTTVTVSS
 7 MOVH IIIB.CON  ARLGYYGYFGSSXXXYWXXAYWGQGTTVTVSS
 8 MOVH IIIC.CON  TTGFVPXXXXXXXXXXXXXXAYWGQGTLVTVSS
10 MOVH VA.CON    ARSNYYGGSYYFFXXXXXFDYWGQGTTLTVSS
11 MOVH VB.CON    AVRVISRYFXXXXXXXXXXDGWGQGTLV
     CONSENSUS    ----------------------WG-GT--TVSS
```

FIG. 6. Consensus sequences of the murine (a) V_H and (b) V_K gene families are listed in the single-letter code. The amino acid positions that are conserved among all V_H/V_K gene families are outlined in the bottom lines.

```
      30        40        50
/TGDSITSDYWNXXWIRQFPGNKLEWM
/SGFSLTSYGVHXSWVRQPPGKGLGTL
ASGYTFTDYYMXXXWVKQSHGKSLEWI
ASGYTFTSYWMHXXWVKQRPGQGLEWI
ASGFNIKDTYMHXXWVKQRPEQGLEWI
TSGFTFSDFYMEXXWVRQPPGKRLEWI
ASGFDFSRYWMSXXWVRQAPGKGLEWI
ASGFTFSNYWMNXXWVRQSPEYGLEWV
ASGYTFTSYGINXXWVKQRPGQGLEWI
ATGYTFSSYGLYXXWVRQAPGQGLEXX
--G----------W--------L---

      80        90        100
ITRDTSKNQFFLQLNSVTTEDTATYYC
ISKDNSKSQVFLKMNSLQTDDTAMYYC
TLTVDKSSSTAYMQLNSLTSEDSAVYYC
TLTVDKSSSTAYMQLSSLTSEDSAVYYC
TITADTSSNTAYLQLSSLTSEDTAVYYC
TVSRDTSQSILYLQMNALRAEDTAIYYC
TISRDNAKNTLYLQMSYVRSEDTALYYC
TISRDDSYSSVYLQMNNLRAEDTGIYYC
TLTVDKSSSTAYMQLRSLTSEDSAVYFC
TITADESTNTAYMELSSLRSEDTAVYFC
----D---------------D---Y-C

      130       140       150
WGQGTTXTVSS
WGQGTLVTVSS
WGQGTTVTVSS
WGQGTTLTVSS
WGQGTSVTVSS
WGAGTTVTVSS
WGQGTTVTVSS
WGQGTLVTVSS
WGQGTTLTVSS
WGQGTLV
WG-GT--TVSS
```

and (b) V_K gene families are listed in the are conserved among all V_H/V_K gene

b

```
                    10        20        30        40        50
1  MOVK I.CON       DIVMTQSPSSLAVSAGEKVTXSCTASESLYSSKHKVHYLAWYQKKPEQS
2  MOVK II.CON      DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSXNGNTYLNWYLQKPGG
3  MOVK III.CON     DIVLTQSPASLAVSLGQRATISCRASESVDXXXYGNSFMHWYQQKPGQPP
4  MOVK IV.CON      EXVLTQSPAIMAASPGEKVTMTCXASSSXXXXXVSSSYLHWYQQKPGASP
5  MOVK V.CON       DIQMTQSPSSLSASLGDRVTITCRASQXXXXXXDISNYLNWYQQKPGGTP
6  MOVK VI.CON      QIVLTQSPAIMSASPGEKVTMTCSASSXXXXXXXSVSYMHWYQQKSGTSP
7  MOVK.MBR         DIQLTQSPPSLTVSVGERVTISCKSNQNLLWSGNRRYCLGWHQWKPGQTP
     CONSENSUS      ----TQ-P-----S-G------C-----------------W---K----P

                    60        70        80        90        100
1  MOVK I.CON       KLLIYGASNRYIGVPDRFTGSGSGTDFTLTISSVQVEDLTHYYCAQFYSY
2  MOVK II.CON      KLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGTHV
3  MOVK III.CON     KLLIYAASNLESGVPARFSGSGSGTDFTLNIHPVEEDDAATYYCQQSNED
4  MOVK IV.CON      KLXIYXTSNLASGVPARFSGSGSGTSYSLTISSXEAEDDATYYCQQWSGY
5  MOVK V.CON       KLLIYYASRLHSGVPSRFSGSGSGTDYSLTISSLEZEDIATYFCQQGNSL
6  MOVK VI.CON      KRWIYDTSKLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSN
7  MOVK.MBR         TPLITWTSDRFSGVPDRFIGSGSVTDFTLTISSVQAEDVAVYFCQQHLDL
     CONSENSUS      ---I---S----GVP-RF-GSGS-T---L-I------D---Y-C-Q----

                    110       120       130       140       150
1  MOVK I.CON       PXXXXXXLTFGAGTKLELYRX
2  MOVK II.CON      PXXXXXXYTFGGGTKLEIKRA
3  MOVK III.CON     PXXXXXXXTFGGGTKLEIKRA
4  MOVK IV.CON      PFXXXXXXTFGXGTKLEIKRX
5  MOVK V.CON       PXXXXXXRTFGGGTKLEIXKRA
6  MOVK VI.CON      PPMXXXXLTFGAGTKLELKRX
7  MOVK.MBR         PXXXXXXYTFGGGTKLEI
     CONSENSUS      P--------TFG-GTKLE----A
```

FIG. 6b.

	V_H	V_K
FR 1	1-30	1-23
CDR1	31-35	24-34
FR2	36-49	35-49
CDR2	50-65	50-56
FR3	66-94	57-88
CDR3	95-102	89-97
FR4	103-113	98-107

FIG. 7. Definition of CDR regions according to Kabat *et al.*[14]

```
                    10        20        30   35ab   40
MOVH IA     EVQLQESGPSLVKPSQTLSLTCSVTGDSITSDYWNXXWIRQFPGNKLEWM
BW431/26VH  QVQLQESGPDLVKPSQSLSLTCTVTGYSITSGYSWH-WIRQFPGNKLEWM
            *   *   *   *  *
              52abc     60        70        80 abc      90
MOVH IA     GYISNXXYSGSTYYNPSLYSRISITRDTSKNQFFLQLNSVTTEDTATYYC
BW431/26VH  GYI----YSGITNYNPSLKSRISITRDTSKNQFFLQLNSVTTEDTATYYC


                  100abcdefghijk      110
MOVH IA     ARLYGYYRGDEEDYYAMAFDYWGQGTTXTVSS
BW431/26VH  AR---------EDYDYHWFDVWGAGTTVTVSS
                           *
```

FIG. 8. Comparison of BW431/26 V_H sequence with the mouse V_H IA (MOVH IA) consensus sequence. Relevant framework differences are marked with an asterisk. The differences are as follows: EQ1, SD10, TS17, ST23, DY27, QA105. X represents amino acids that are variable in the consensus sequence. These are not considered as exchanges. Dashes in the BW431/26 sequence are introduced to accommodate for the variable length of the CDRs.

In the case of the BW431/26 antibody, for V_H the exchange of a phenylalanine for a serine at position 27, and that of an isoleucine for a phenylalanine at position 29 in the NEW V_H gene have been considered as additional framework exchanges. With the exchange of the amino acid at position 27 from serine to phenylalanine we followed Riechmann's strategy for the humanization of the CAMPATH 1 antibody.[13] Phe-27 is present in most human V_H domains and may play an important role in positioning CDR1. Since Phe-27 is changed to serine in the NEW V_H domain it seems necessary to introduce Phe-27 into the humanized V_H.

Phe-29 of NEW in the immediate vicinity of CDR1 contains an aro-

```
                   10        20        30   35ab   40
HuVH.NEW    QVQLQESGPGLVRPSQTLSLTCTVSGSTFSDYYSTXXWVRQPPGRGLEWI
HuVH.CON2   XVTLRESGPXLVKPTETLTLTCTVSGFSLSTXGMXVGWIRQPPGKXLEWL
              * *                   * * *

              52abc     60        70          82abc      90
HuVH.NEW    GYVFYHGTSDTDTPLRSXXXRVTMLVDTSKNQFSLRLSSVTAADTAVYYC
HuVH.CON2   ARINXXXWDDDKYYSTSLRSRLTISYDTSKNQVVLXXXXXDPXDTATYYC
             * *   **  *****     *  **      **      **    *

                 100abcdefghijk     110
HuVH.NEW    ARNLIAGCIXXXXXXXXXXDVWGQGSLVTVSS
HuVH.CON2   ARRXPRXXXGDXGXYXXAFDVWGQGTTVTVSS
              * **                 *
```

FIG. 9. Comparison of the NEW V_H sequence with the human V_H II (HuVH.CON2) family consensus sequence. The NEW V_H sequence is based on Ref. 13. Framework differences are marked with an asterisk. The differences are as follows: QT3, QR5, SF27, FL29, DT31, YR50, FN52, TW53, SD54, DK57, TY58, PY59, LS60, RT61, VL67, LS70, VY71, FV78, SV79, TD83, AP84, VT89, NR95, IP97, AR98, LT109.

```
     30   35ab   40
VTGDSITSDYWNXXWIRQFPGNKLEWM
VTGYSITSGYSWH-WIRQFPGNKLEWM
   *
70        80 abc       90
ITRDTSKNQFFLQLNSVTTEDTATYYC
ITRDTSKNQFFLQLNSVTTEDTATYYC


   110
QGTTXTVSS
AGTTVTVSS
*
```

ice with the mouse V_H IA (MOVH IA) es are marked with an asterisk. The differ- 27, QA105. X represents amino acids that iot considered as exchanges. Dashes in the ite for the variable length of the CDRs.

ody, for V_H the exchange of a 7, and that of an isoleucine for a V_H gene have been considered as he exchange of the amino acid at e we followed Riechmann's strat- IPATH 1 antibody.[13] Phe-27 is d may play an important role in anged to serine in the NEW V_H Phe-27 into the humanized V_H. icinity of CDR1 contains an aro-

```
     30   35ab   40
TVSGSTFSDYYSTXXWVRQPPGRGLEWI
TVSGFSLSTXGMXVGWIRQPPGKXLEWL
   * * *

 70          82abc       90
TMLVDTSKNQFSLRLSSVTAADTAVYYC
TISYDTSKNQVVLXXXXXDPXDTATYYC
  **       **       **    *

   110
GQGSLVTVSS
GQGTTVTVSS
   *
```

e with the human V_H II (HuVH.CON2) ice is based on Ref. 13. Framework differ- s are as follows: QT3, QR5, SF27, FL29, , PY59, LS60, RT61, VL67, LS70, VY71, R98, LT109.


```
                10        20        30   35ab   40
HuVH II     -VTLRESGPXLVYPTETLTLTCTVSGFSLSTXGMXVGWIRQPPGKXLEWL
MOVH IA     EVQLQESGPSLVKPSQTLSLTCSVTGDSITSDYWNXXWIRQFPGNKLEWM
              * *       * *         *               * *

              52abc      60        70        80 abc       90
HuVH II     ARINXXXWDDDKYYSTSLRSRLTISYDTSKNQVVLXXXXXDPXDTATYYC
MOVH IA     GYISNXXYSGSTYYNPSLYSRISITRDTSKNQFFLQLNSVTTEDTATYYC
                                    *      **       ***

                 100abcdefghijk       110
HuVH II     ARRXPRXXXGDXGXYXXAFDVWGQGTTVTVSS
MOVH IA     ARLYGYYRGDEEDYYAMAFDYWGQGTTXTVSS
              * **   ** *       *
```

FIG. 10. Comparison of the acceptor (human V_H II; HuVH II) and the donor mouse V_H IA; MOVH IA) consensus sequences. Relevant differences in the framework regions are marked with an asterisk. Differences between donor and acceptor consensus sequences are as follows: DEL E1, TQ3, RQ5, YK13, EQ16, FD27, PF40, KN43, YR71, VF78, VF79, XQ81, XS82C, XV83, DT84, PT85, XE86, RL95, XY96 (X = T, C, V, P, Q, H, R, L, N), PG97, RY98, XY99 (X = P, Q, V, R, M, T, G), DE100C, XE100D (X = Y, L, M, G, V), GD100E, XA100H (X = N, S, D, Y), XM100I (X = S, D, G), VY102.

```
                10        20     27abcdef 30        40
MOVK VI     QIVLTQSPAIMSASPGEKVTMTCSASSXXXXXXXSVSYMHWYQQKSGTSP
BW431/26VK  --------AILSASPGEKVTMTCRASSXXXXXXXSVSYMHWYQQKPGSSP
                                                      *

              50        60        70        80        90
MOVK VI     KRWIYDTSKLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSN
BW431/26VK  KPWIYATSNLASGVPARFSGSGSGTSYSLTIIRVEAEDAATYYCQQWSSN
              *                             **

           95abcdef  100   106a
MOVK VI     PPMXXXXLTFGAGTKLELXKRX
BW431/26VK  PXXXXXXLTFGAGTKLEIX---
```

FIG. 11. Comparison of the 431/26 V_K sequence with the mouse V_K VI (MOVK VI) family consensus sequence. The relevant differences in the framework regions are marked with an asterisk. The differences are as follows: SP40, RP46, SI76, SR77.

```
                  10        20     27abcdef 30        40
HuVKCON SUB1    DIQMTQSPSSLSASVGDRVTITCRASQSVXXSXDISSYLNWYQQKPGKAP
HuVK REI        DIQMTQSPSSLSASVGDRVTITCQASQ------DISDYLNWYQQKPGKAP

                  50        60        70        80        90
HuVKCON SUB1    KLLIYXASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSL
HuVK REI        KLLIYEASNLQAGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQYQSL
                                                    *

               95abcdef  100   106a
HuVKCON SUB1    PXXYDXXYTFGQGTKVEIXKRT
HuVK REI        P------YTFGQGTKVEI-KR-
```

FIG. 12. Comparison of the human acceptor sequence REI with the consensus sequence of the human V_K I family (HuVKCON SUB1). The relevant framework difference is marked with an asterisk; the difference is FI83.

```
                      10        20     27abcdef 30       40
HuVKCON SUB1   DIQMTQSPSSLSASVGDRVTITCRASQSVXXSXDISSYLNWYQQKPGKAP
   MOVK VI     QIVLTQSPAIMSASPGEKVTMTCSASSXXXXXXXSVSYMHWYQQKSGTSP
                 *     **    *                              * **

                    50        60        70        80        90
HuVKCON SUB1   KLLIYXASSLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSL
   MOVK VI     KRWIYDTSKLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSN
                **          *         *         * *

                95abcdef  100   106a
HuVKCON SUB1    PXXYDXXYTFGQGTKVEIXKRT
   MOVK VI      PPMXXXXLTFGAGTKLELXKRX
                          *
```

FIG. 13. Comparison of the consensus sequences of the acceptor (HuVKCON SUB1) and donor (MOVK VI) V_K families. Relevant differences in the framework regions are marked with an asterisk. The differences are as follows: QV3, SA9, SI10, VP15, PS41, KT43, AS44, LR47, LW48, SA60, DS70, PA80, FA83, QA100.

matic ring and is larger than Ile-29 of BW431/26. Therefore it might interact with CDR1 side chains in a different way than does Ile-29, so we decided to use Ile-29 in the humanized MAb. For the light chain no additional exchanges were necessary.

On the basis of these data the oligonucleotides for the mutagenesis of the human V genes were designed. The oligonucleotides span the CDR coding region, including additional exchanges in the adjacent frameworks, and at their 5′ and 3′ regions they contain 12 bp that is complementary to the flanking framework regions of the human V genes. The CDR1 oligonucleotide for the light chain had to be placed differently (Fig. 14).

For the VK CDR1 oligonucleotide the overlap with the frameworks at its 3′ and 5′ ends had to be shortened to 9 b. The full-length (12-b overlap) oligonucleotide exhibited cross-hybridization with framework three sequences and this gave rise to misincorporation during mutagenesis.

Mutagenesis of Human V Genes

The mouse CDRs are placed into the human frameworks by oligonucleotide-directed mutagenesis. The mutagenesis of the human V genes can be performed with any mutagenesis system available. We use the gapped duplex (gd) DNA method and the pMa/c phasmid vectors described by Stanssens and colleagues.[26] The gapped duplex method routinely yields a

[26] P. Stanssens, C. Opsomer, Y. M. McKeown, W. Kramer, M. Zabeau, and H.-J. Fritz, *Nucleic Acids Res.* **17**, 4441 (1989).

```
)      27abcdef 30        40
FITCRASQSVXXSXDISSYLNWYQQKPGKAP
FMTCSASSXXXXXXXSVSYMHWYQQKSGTSP
                          * **


      70         80         90
GSGSGTDFTLTISSLQPEDFATYYCQQYNSL
GSGSGTSYSLTISSMEAEDAATYYCQQWSSN
       *          * *

KRT
KRX
```

nces of the acceptor (HuVKCON SUB1) and rences in the framework regions are marked QV3, SA9, SI10, VP15, PS41, KT43, AS44,)0.

of BW431/26. Therefore it might lifferent way than does Ile-29, so we ıized MAb. For the light chain no

gonucleotides for the mutagenesis of The oligonucleotides span the CDR changes in the adjacent frameworks, ıtain 12 bp that is complementary to e human V genes. The CDR1 oligo- ɔe placed differently (Fig. 14). e the overlap with the frameworks at to 9 b. The full-length (12-b overlap) idization with framework three se- rporation during mutagenesis.

) the human frameworks by oligonu- ıutagenesis of the human V genes can system available. We use the gapped pMa/c phasmid vectors described by ɔed duplex method routinely yields a

vn, W. Kramer, M. Zabeau, and H.-J. Fritz,

VK - CDR1

5′ CTG GTA CCA **GTG CAT GTA ACT TAC ACT CGA GCT GGT** **ACT** ACA GGT GAT 3′

VK - CDR2

5′ GCT TGG CAC ACC **AGA AGC CAG GTT GGA TGT GCT** GTA GAT CAG CAG 3′

VK - CDR3

5′ CCC TTG GCC GAA **CGT GGG ATA ACT ACT CCA CTG ATG** GCA GTA GTA GGT 3′

VH - CDR1

5′ CTG TCT CAC CCA **GTG CCA GCT ATA ACC ACT** GCT **GAT** GGT **GAA** GCC AGA CAC GGT 3′

VH - CDR2

5′ CAT TGT CAC TCT **ACT TTT GAG AGA GGG GTT GTA GTT AGT** **GAT ACC ACT GTA CTG TAT GTA** TCC AAT CCA CTC 3′

VH - CDR3

5′ GCC TTG ACC CCA **GAC ATC GAA GTA CCA GTG GTA ATC** **ATA GTC TTC** TCT TGC ACA ATA 3′

FIG. 14. Oligonucleotides for the CDR exchange mutagenesis. The bases originated from the BW431/26 sequence and the codon for Phe-27 in V_H are printed in bold letters.

larger percentage of recombinant clones with all three CDRs mounted in a single step (5–20%), as compared with classical M13 mutagenesis.[13] The template for mutagenesis of the V_H gene is the human myeloma protein NEW and for the V_L gene it is human myeloma protein REI, or any CDR-grafted version of these genes. We used the humanized versions containing the CDR regions of the anti-lysozyme MAb D1.3[27] (Fig. 15a and b).

[27] M. Verhoeyen, C. Milstein, and G. Winter, *Science* **239**, 1534 (1988).

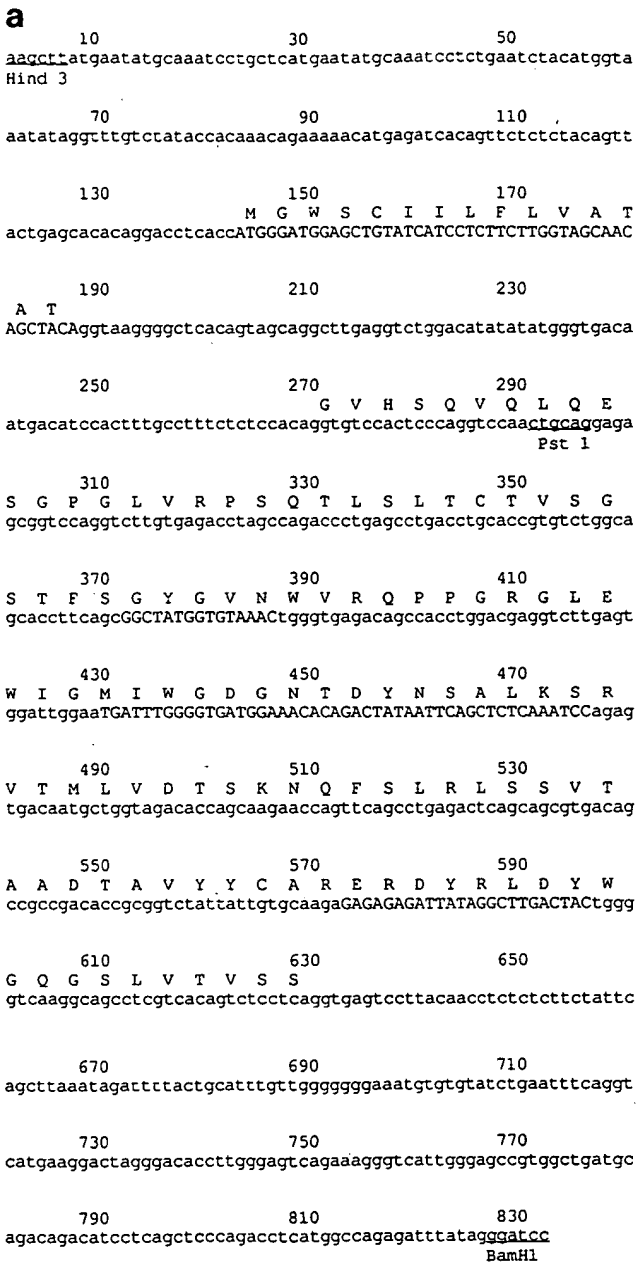


FIG. 15. Nucleotide sequences of the (a) acceptor V_H (anti-Lys-24) and (b) V_K gene (anti-Lys-18) segments of the pMc vectors used for CDR exchange mutagenesis. The amino acid sequences are printed in single-letter code on top of the central base of a triplet. The CDR regions are given in capital letters.

```
                50
ıatcctctgaatctacatggta

               110
ɜaccacagttctctctacagtt

               170
I  I  L  F  L  V  A  T
TCATCCTCTTCTTGGTAGCAAC

               230

ctggacatatatatgggtgaca

               290
 S  Q  V  Q  L  Q  E
:tcccaggtccaactgcaggaga
                  Pst 1

               350
 L  T  C  T  V  S  G
:ctgacctgcaccgtgtctggca

               410
 P  P  G  R  G  L  E
ɔccacctggacgaggtcttgagt

               470
 N  S  A  L  K  S  R
TAATTCAGCTCTCAAATCCagag

               530
 L  R  L  S  S  V  T
cctgagactcagcagcgtgacag

               590
 D  Y  R  L  D  Y  W
AGATTATAGGCTTGACTACtggg

               650

:cttacaacctctctcttctattc

               710
ıatgtgtgtatctgaatttcaggt

               770
ɟtcattgggagccgtggctgatgc

               830
ıgatttatagggatcc
          BamH1
```

ɔtor V_H (anti-Lys-24) and (b) V_K gene CDR exchange mutagenesis. The amino top of the central base of a triplet. The

b

```
          10                  30                  50
aagcttatgaatatgcaaatcctctgaatctacatggtaaatataggtttgtctatacca
Hind 3

          70                  90                 110
caaacagaaaaacatgagatcacagttctctctacagttactgagcacacaggacctcac

         130                 150                 170
  M  G  W  S  C  I  I  L  F  L  V  A  T  A  T
cATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAggtaaggggctcac

         190                 210                 230
agtagcaggcttgaggtctggacatatatatgggtgacaatgacatccactttgcctttc

         250                 270                 290
    G  V  H  S  D  I  Q  M  T  Q  S  P  S  S  L  S  A
tctccacaggtgtccactccgacatccagatgacccagagcccaagcagcctgagcgcca

         310                 330                 350
S  V  G  D  R  V  T  I  T  C  R  A  S  G  N  I  H  N  Y  L
gcgtgggtgacagagtgaccatcacctgtAGAGCCAGCGGTAACATCCACAACTACCTGG

         370                 390                 410
A  W  Y  Q  Q  K  P  G  K  A  P  K  L  L  I  Y  Y  T  T  T
CTtggtaccagcagaagccaggtaaggctccaaagctgctgatctacTACACCACCACCC

         430                 450                 470
L  A  D  G  V  P  S  R  F  S  G  S  G  S  G  T  D  F  T  F
TGGCTGACggtgtgccaagcagattcagcggtagcggtagcggtaccgacttcaccttca

         490                 510                 530
T  I  S  S  L  Q  P  E  D  I  A  T  Y  Y  C  Q  H  F  W  S
ccatcagcagcctccagccagaggacatcgccacctactactgcCAGCACTTCTGGAGCA

         550                 570                 590
T  P  R  T  F  G  Q  G  T  K  V  E  I  K  R
CCCCAAGGACGttcggccaagggaccaaggtggaaatcaaacgtgagtagaatttaaact

         610
ttgcttcctcagttggatcc
          BamH1
```

FIG. 15b.

Preparation of Template and Gapped Duplex DNA

The human V genes were cloned into the *Hin*dIII and *Bam*HI sites of modified pMc and pMa phasmids. In order to use the mutagenic oligonucleotides as designed, the polylinkers of pMa/c had to be inverted.

For the preparation of template DNA and gapped duplex DNA we follow the protocol described in Ref. 26. The single-stranded DNA (ssDNA) is prepared from the pMc version of the vector. The double-stranded DNA (dsDNA) is prepared from the pMa version using the restriction enzymes *Xba*I and *Eco*RI to release the V gene insert from the vector.

Mutagenesis

The gdDNA is used as template for the site-specific mutagenesis to exchange the CDR regions. Usually we try to exchange all three CDRs in one round of mutagenesis. A typical experiment is as follows.

Generation of gdDNA. ssDNA (0.5 p*M*: 770 ng for V_L and 805 ng for V_H) is mixed with 0.1 p*M* dsDNA (*Eco*RI/*Xba*I cut) + 5 μl 1.5 *M* KCl in 100 m*M* Tris, pH 7.5, + H_2O to 40 μl. Incubate at 100° for 4 min and then at 65° for 10 min in water baths. Use 8 μl for gel analysis.

Annealing and Polymerase Reaction. To 8 μl of gdDNA, add 3 μl of each mutagenic oligonucleotide (10 μ*M*/ml, kinased). Incubate at 68° for 5 min and at room temperature for 15 min. Add the following:

Fill-in buffer (10×): 500 m*M* NaCl, 100 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 10 m*M* DTT, 10 m*M* dNTPs; 4 μl
T4 ligase (2U), 2 μl
Klenow polymerase (2.5 U), 0.5 μl
H_2O to 40 μl

Incubate at room temperature for 45 min.

Selection and Screening for Mutants. Transform 5 μl of the reaction mixture into WK6mutS[28] bacteria and plate aliquots on 1× YT[29] (yeast Tryptone)-Amp plates (200 μg/ml ampicillin) and YT-chloramphenicol plates (30 μg/ml chloramphenicol) to determine the efficiency of transformation. The remaining transformation mixture is used to inoculate 10 ml LB[29] (Luria-Bertani) medium (200 μg/ml ampicillin). Bacteria are grown overnight to allow segregation of strands. Plasmid DNA is isolated[29] and used to transform WK6 bacteria[28] selecting for Amp resistance (200 μg/ml ampicillin).

The resulting colonies are screened with the CDR3 oligonucleotides described in Fig. 4 using standard colony hybridization methods.[29] From the colonies hybridizing with the probe, 1.5-ml cultures are grown (YT, 200 μg/ml ampicillin), DNA is prepared and the nucleotide sequence determined using the V_H and V_L gene sequencing oligonucleotides (Fig. 4). Five to 20% of the clones hybridizing with the CDR3 mutagenic oligonucleotide have all three CDRs placed properly.

For antibody BW431/26 the sequences of the humanized V gene versions were confirmed (Fig. 16).

[28] R. Zell and H.-J. Fritz, *EMBO J.* **6**, 1809 (1987).
[29] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

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M: 770 ng for V_L and 805 ng for
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Incubate at 100° for 4 min and
e 8 μl for gel analysis.
To 8 μl of gdDNA, add 3 μl of
ıl, kinased). Incubate at 68° for 5
. Add the following:

m*M* Tris-HCl, pH 7.5, 100 m*M*
s; 4 μl

l.
Transform 5 μl of the reaction
ılate aliquots on 1× YT[29] (yeast
cillin) and YT-chloramphenicol
ermine the efficiency of transfor-
ıixture is used to inoculate 10 ml
l ampicillin). Bacteria are grown
. Plasmid DNA is isolated[29] and
ıg for Amp resistance (200 μg/ml

with the CDR3 oligonucleotides
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1.5-ml cultures are grown (YT,
and the nucleotide sequence de-
ıencing oligonucleotides (Fig. 4).
:h the CDR3 mutagenic oligonu-
erly.
es of the humanized V gene ver-

olecular Cloning: A Laboratory Manual."
rbor, New York, 1982.

BW431/26VHhum

```
        10                  30                  50
Q V Q L Q E S G P G L V R P S Q T L S L
caggtccaactgcaggagagcggtccaggtcttgtgagacctagccagaccctgagcctg

        70                  90                  110
T C T V S G F T I S S G Y S W H W V R Q
acctgcaccgtgtctggcTTCaccATCagcAGTGGTTATAGCTGGCACtgggtgagacag

       130                 150                 170
P P G R G L E W I G Y I Q Y S G I T N Y
ccacctggacgaggtcttgagtggattggaTACATACAGTACAGTGGTATCACTAACTAC

       190                 210                 230
N P S L K S R V T M L V D T S K N Q F S
AACCCCTCTCTCAAAAGTagagtgacaatgctggtagacaccagcaagaaccagttcagc

       250                 270                 290
L R L S S V T A A D T A V Y Y C A R E D
ctgagactcagcagcgtgacagccgccgacaccgcggtctattattgtgcaagaGAAGAC

       310                 330                 350
Y D Y H W Y F D V W G Q G S L V T V T V
TATGATTACCACTGGTACTTCGATGTCtggggtcaaggcagcctcgtcacagtcacagtc
```

BW431/26VKhum

```
        10                  30                  50
G V H S D I Q M T Q S P S S L S A S V G
ggtgtccactccgacatccagatgacccagagcccaagcagcctgagcgccagcgtgggt

        70                  90                  110
D R V T I T C S T S S S V S Y M H W Y Q
gacagagtgaccatcacctgtAGTACCAGCTCGAGTGTAAGTTACATGCACtggtaccag

       130                 150                 170
Q K P G K A P K L L I Y S T S N L A S G
cagaagccaggtaaggctccaaagctgctgatctacAGCACATCCAACCTGGCTTCTggt

       190                 210                 230
V P S R F S G S G S G T D F T F T I S S
gtgccaagcagattcagcggtagcggtagcggtaccgacttcaccttcaccatcagcagc

       250                 270                 290
L Q P E D I A T Y Y C H Q W S S Y P T F
ctccagccagaggacatcgccacctactactgcCATCAGTGGAGTAGTTATCCCACGttc

       310                 330
G Q G T K V E I K R
ggccaagggaccaaggtggaaatcaaacgt
```

FIG. 16. Nucleotide sequences of the humanized V_H (top) and V_K (bottom) of MAb BW431/26. The sequences start with the codon for the first amino acids of the mature heavy and light chains. The human framework sequences are printed in lower-case letters while the murine CDR sequences and the murine framework sequences that have been transferred to the human V genes are printed in capital letters. The amino acids are given in the single-letter code and are printed on top of the first base of the codon.

Expression of Humanized MAb

The humanized V genes were cloned into separate eukaryotic expression vectors that contain Ig constant-region genes for the heavy and light chain, respectively (Fig. 3). The resulting V_H and V_L expression vectors were subsequently transfected into BHK cells (ATCC CCL10) using the calcium phosphate precipitation technique with methotrexate and G418 as selection markers.[30] We were able to isolate clones that produce up to 15 μg/ml antibody product in serum-free roller bottle cultures without prior amplification.

Characterization of Humanized MAb

The humanized BW431/26 binds to purified CEA and to CEA on tissue sections. Competition experiments with the original mouse MAb reveal that the affinity of the reshaped antibodies, although it has not yet been determined exactly, closely resembles that of the mouse MAb, which is approximately 1×10^{-10} liter/mol.

Anti-idiotypic antibodies (anti-id) against the murine BW431/26 MAb were tested for their binding to the humanized version. Surprisingly, 17 out of 18 anti-id tested bind the humanized version as well as the mouse MAb; binding of one anti-id is less efficient but still detectable. This finding suggests a close immunological relationship between the variable domains of the mouse and the humanized BW431/26 (K. Bosslet *et al.*, unpublished data, 1991).

A comparison of the framework amino acid sequences of the donor (the murine MAb BW431/26) V_H and V_L with the acceptor V_H (human NEW) and V_L (human REI) shows a high degree of sequence similarity (70% for V_H and 68% for V_K). The homologies are clustered in the regions close to the CDRs, especially in the light chain domains. Even in positions where the amino acid sequences differ from each other the amino acid exchanges are often conservative. This may have contributed to the successful humanization of the BW431/26 antibody. The crystal structures of the REI and NEW antibodies are known and we use their respective V regions as general acceptors for the CDRs of any murine MAb. Computer modeling is used for refinement to identify potential framework amino acids in the mouse antibody that might interact with the CDRs or directly with antigen and these amino acids are transferred to the human frameworks along with the CDRs. Using the reshaping scheme described here we estimate the success rate for humanizing any particular monoclonal antibody to be approximately 80%.

[30] M. Wirth, J. Bode, G. Zettlmeissl, and H. Hauser, *Gene* **73**, 419 (1988).

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ı genes for the heavy and light
V_H and V_L expression vectors
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with methotrexate and G418 as
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roller bottle cultures without

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transferred to the human frame-
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; any particular monoclonal anti-

r, *Gene* **73**, 419 (1988).

Queen *et al.*[31] have reshaped an antibody by selecting human acceptor V domains from the Kabat database to match the framework sequences of the murine donor V domains as closely as possible. This alternative reshaping strategy raises the possibility that any human antibody framework can be used as acceptor for the murine CDRs. Humanization of murine MAbs is a technique that is now widely applied to MAbs of potential use in diagnosis and therapy in humans. Whether one of the reshaping schemes will offer any particular advantage in successful humanization of MAbs will become clearer as the number of reshaped antibodies increases.

Acknowledgments

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[31] C. Queen, W. P. Schneider, H. E. Selick, P. W. Payne, N. F. Landolfi, J. F. Duncan, N. M. Avdalovitc, M. Levitt, R. P. Junghans, and T. A. Waldmann, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 10029 (1989).

[6] Molecular Modeling of Antibody Combining Sites

By ANDREW C. R. MARTIN, JANET C. CHEETHAM, and ANTHONY R. REES

Introduction

Both the variable and constant domains of an antibody Fab consist of two twisted antiparallel β sheets which form a β-sandwich structure. The constant regions have three- and four-stranded β sheets arranged in a Greek key-like motif,[1] while variable regions have a further two short β strands producing a five-stranded β sheet.

The two β sheets of the variable domain are inclined at 30° to one another,[2] with a conserved disulfide bridge in each domain linking the two β sheets. Lesk and Chothia[3] have shown the relative orientation of the two

[1] J. S. Richardson, *Adv. Protein Chem.* **34**, 168 (1981).
[2] C. Chothia and J. Janin, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4146 (1981).
[3] A. M. Lesk and C. Chothia, *J. Mol. Biol.* **160**, 325 (1982).